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Green tea extract modulates lithium-induced thyroid follicular cell damage in rats

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Abstract:

Background: The aim of the current work was to clarify the modulation role of green tea extract (GTE) over structural and functional affection of the thyroid gland after long term use of lithium carbonate (LC). The suggested underlying mechanisms participating in thyroid affection were researched.

Materials and methods: Twenty-four Sprague-Dawley adult albino rats were included in the work. They are divided into three groups (control, LC, and concomitant LC + GTE). The work was sustained for 8 weeks. Biochemical assays were achieved (thyroid hormone profile, IL-6). Histological, histochemical (PAS) and immunohistochemical (caspase-3, TNF-α, PCNA) evaluations were done. Oxidative/antioxidative markers (MDA / GSH, SOD) and western blot evaluation of the Bcl2 family were done.

Results: LC induced hypothyroidism (decrease T3, T4/increase TSH). The follicles were distended, others were involuted. Some follicles were disorganized, others showed detached follicular cells. Apoptotic follicular cells were proved (Bax and caspase-3 increased, Bcl2 decreased, Bax/Bcl2 ratio increased). The collagen fibers' content and proinflammatory markers (TNF-α and IL-6) increased. The proliferative nuclear activity was supported by increase

expression of PCNA. Oxidative stress was established (increase MDA/decrease GSH, SOD). With the use of GTE, the thyroid hormone levels increased, while the TSH level decreased. Apoptosis is improved as Bax decreased, Bcl2 increased, and Bax/Bcl2 ratio was normal. The collagen fibers' content and proinflammatory markers (TNF-α and IL-6) decreased. The expression of PCNA and caspase-3 were comparable to the control group. The oxidative markers were improved (decrease MDA/increase GSH, SOD).

Conclusions: In conclusion, prolonged use of LC results in hypothyroidism, which is accompanied by structural thyroid damage. LC induced thyroid damage through oxidative stress that prompted sterile inflammation and apoptosis. With the use of GTE, the thyroid gland achieved its structure and function. The protecting role of GTE is through antioxidant, antifibrotic, anti-inflammatory, and antiproliferative effects.

Key word: lithium carbonate, green tea extract, thyroid damage, oxidative stress, inflammation, apoptosis

INTRODUCTION

Lithium (Li) and its salts (as lithium carbonate) (LC) are commonly used for the treatment of numerous psychiatric illnesses (Focosi et al., 2009). It is used in the therapy of mood instability (bipolar disorder) and has a prospective role in the therapy of mania and depression (Kalantari et al., 2015). It precludes mood swings in patients with manic-depressive disorder (Bjelakovic et al., 2007). Although it is a unique drug, prolonged treatment with therapeutic levels may cause multisystem toxicity (B N et al., 2013). It can disturb the function of the heart, liver, kidney, testes, and gastrointestinal system. Adding, it can induce diabetes insipidus, acneform eruptions, renal toxicity, and brain damage (Singer et al., 1972, Lazarus and Bennie, 1972, Gosselin et al., 1984, Thakur et al., 2003).

The concentration level of Li in the thyroid gland is 3-4 times that in plasma (Berens et al., 1970). Li influences the function of the thyroid gland, either directly or indirectly via, the hypothalamic- pituitary- thyroid axis (Lazarus, 2009). It interferes with thyroid functions at the stage of hormonal secretion (George and Joshi, 2007). It competes for iodide transport, increases thyroidal radioiodine retention, and decreases deiodination from T4 to T3 (Lazarus, 2009). It may cause hypothyroidism , goiter , or infrequently thyrotoxicosis (George and Joshi, 2007).

Some researchers described lithium-induced hypothyroidism was associated with oxidative stress (Toplan et al., 2013). Furthermore, others described the alteration of the thyroid gland at the cellular and subcellular levels (Valle et al., 1993).

Green tea (GT) is one of the common beverages in the globe. Its chief components are the polyphenol (catechins) (Singhal et al., 2017). GT catechins are Epigallocatechin, Epigallocatechin-3-gallate (EGCG), Epicatechin, and Epicatechin-3-gallate (Galati et al., 2006). Moreover, GT contains caffeine, quercetin, chlorogenic acid, garlic acid, myricetin, and kaempferol (Singhal et al., 2017). GT polyphenols have protective roles against neurodegenerative diseases, cancer, heart disease, lung damages, and diabetes (Higdon and Frei, 2003, Singhal et al., 2017).

The mechanism through which LC induces thyroid damage at the cellular level is not clearly understood and not enough studied. Furthermore, no works have investigated the protective role of GTE over such damage. So, the present work intended to clarify the modulation role of GTE over structural and functional affection of thyroid gland after long term use of LC. The suggested underlying mechanisms participating in thyroid affection were investigated.

MATERIALS AND METHODS

Animals

Twenty-four Sprague-Dawley adult albino rats were included in the work. They were housed in a dark /light cycle (12/12-h), humidity (50−60%) and temperature (25 ± 1°C). The study was completed in the Experimental Animal Centre, Faculty of Medicine, Cairo University.

Experimental design

The rats were distributed into three groups (eight in each group): control, LC, and GTE (concomitant LC + GTE).

Test materials

LC was obtained in the structure of tablets (400 mg) (Nile Co. for Pharmaceuticals and Chemical Industries, Egypt). Tablets were liquified in distilled water and given through an intragastric tube in a dose of 30 mg/kg/day for eight weeks (El-Mahalaway and El-Azab, 2019).

GTE was obtained in the structure of tablets (200 mg) (Techno-med Group Co., Egypt). Tablets were melted in distilled water and given orally in a dose of 150 mg/ kg/day for eight weeks (Hamdy et al., 2012).

I. General health profile

Food and water intake, motility, and health condition were recorded daily. Bodyweight (BW) was documented at the start and end of the study.

II. Biochemical assay

The serum levels of total T3 (TT3), free T3 (FT3), total T4 (TT4), free T4 (FT4) and thyroid-stimulating hormone (TSH) were determined by radioimmunoassay (xh6080, Xi'an). The inflammatory marker IL-6 was assessed by the commercially ELISA kits according to manufacturer's instructions.

III. Tissue sampling

Thyroid was dissected and fixed in 10% formalin saline. The tissue was sectioned every 10th section (5 µm thick). The thyroid tissue extract was prepared according to Gordon et al. (Gordon et al., 1982)

IV. **Light microscopic study:** Hematoxylin & eosin (H &E) and Masson's trichrome stained sections were prepared according to Suvarna et al. (Suvarna et al., 2019)

V. **Histochemical evaluation (PAS stain)** stained sections were prepared according to Suvarna et al. (Suvarna et al., 2019)

VI. Immunohistochemistry(Ramos-Vara et al., 2008)

Paraffin sections were prepared. Then, suitable quantity of serum was added to the sections for 30 min.

i. **Caspase-3:** the sections were incubated with anti-active caspase-3 antibody (Cat #: ab13847, Abcam, Cambridge, MA, USA), then, followed with biotinylated secondary antibody (LSAB kit, Dako Carpentaria, CA, USA). After that, they were incubated with streptavidin HRP (LSAB kit, Dako, Carpentaria, CA, USA), then followed with 3′-Diaminobenzidine (0.05% DAB).

ii. **TNF-α:** the sections were incubated with the primary monoclonal anti-TNF-α antibody (52B83) using dilutions 1:5000 for 36 h at 4^{o}C. Then, they were incubated with biotinylated secondary antibodies for 5 h and then followed by Avidin-Biotin-Peroxidase Complex (ABC). Finally, the immune reaction was visualized with 0.05% DAB.

iii. **Proliferating cell nuclear antigen (PCNA) immunostaining**

PCNA is a helper protein of DNA polymerase enzymes and is used as a typical marker for proliferating cells (Oktay et al., 1995). The immunostaining essential pretreatment was done by boiling for 10 min in 10 mmol/L citrate buffer (catalog number AP 9003) (pH 6) for antigen retrieval. Then, the sections were incubated with the primary antibody (a rabbit polyclonal antibody) (catalog number ab15497, Abcam, Cambridge, UK). Immunostaining was finalized by using the Ultra-vision detection system (catalog number TP-015-HD). The small intestine was applied as a positive control section. The positive reaction appeared as brown nuclear coloration (Oktay et al., 1995).

VII. Oxidative/ antioxidative markers

1. Thyroid lipid peroxidation

Malondialdehyde (MDA) was measured using the method of Buege (D'souza et al., 2012). Briefly, 100 µL serum was diluted with distilled water to 500 µL. 1 mL of TBA–HCl reagent was added to the diluted sample. The reaction mixture was centrifuged, and the supernatant was taken. The optical density was measured spectrophotometrically at 532 nm. The concentration of MDA in the sample was got by plotting the obtained absorbance against the standard graph.

2. Thyroid superoxide dismutase (SOD)

SOD was measured according to the modified Misra and Fridovich's method (Misra and Fridovich, 1972). 10 µL of bovine catalase and 1965 µL of Na2CO3 buffer was added to 5 µL of 10% thyroid homogenate. Then, the mixture was added to 20 µL of 30 mM epinephrine in 0.05% ethanoic acid. The activity of SOD was determined at 37 °C at 480 nm in a spectrophotometer (Model BL 158, ELICO) at 0 minutes and after 3 minutes. The activity of SOD was expressed as the quantity of the enzyme that impedes the oxidation of epinephrine by 50%= 1 U/µg/mg tissue protein.

3. Thyroid glutathione (GSH)

The measurement of GSH was constructed on the reduction of DTNB with GSH to produce a yellow compound. The reduced chromogen was measured at 405 nm. The concentration of the GSH was calculated using the standard curve and expressed /mg of tissue protein (Tipple and Rogers, 2012).

VIII. Morphometric analysis

The content of collagen fibers and the optical density of PAS in colloids and follicular basement membranes were assessed. Adding, the immune expression of TNF-α, caspase-3 positive follicular cell nuclei (%), and the PCNA positive follicular cell nuclei (%) were evaluated using the Leica LAS V3.8 image analyzer computer system (Switzerland).

IX. Western Blot

The homogenized thyroid tissue was added to the ReadyPrep ^{TM} protein extraction kit (Bio-Rad Inc., Catalog #163-2086). 20 μg protein concentration was loaded with an equal volume of 2x Laemmle sample buffer. Primary antibodies of Bcl2 and Bax (Santa Cruz Biotechnology, Inc. catalog numbers: sc-7382 and sc-7480) were diluted in TBST and incubated overnight against the blotted target protein and then followed in the HRP-conjugated secondary antibody (Goat anti-rabbit IgG- HRP-1mg Goat Novus Biologicals) solution for 1 hr at RT. The chemiluminescent substrate (Clarity^{TM} Western ECL substrate Bio-Rad cat#170-5060) was applied to the blot.

X. Statistical analysis

Statistical evaluation was performed using SPSS version 21 (IBM Corporation, Somers, NY, USA) statistical software. Data were stated as means ± SD. Statistical evaluation was done by mean of ANOVA tracked by Bonferroni pairwise comparisons.

RESULTS

I. The general health data

The water and food consumption and health condition were relatively exceptional in all groups. At the beginning of the study, the BW was 160.5±17.1 g. By the end of the experiment, the BW of the LC group increased by 21% compared to the control group. Simultaneous administration of GTE along with LC ameliorates the weight gain (12% decrease) as compared to the LC group. BW of the control and GTE groups were similar (Table 1).

II. Hormonal results

The LC group exhibited a significant decrease in TT3, TT4 (29, 32%) and FT3, FT4 (53, 50 %) compared to the control group. Adding, the TSH level in this group increased by 161 % compared to the control group (Table 2).

With the use of GTE, the serum level of TT3, TT4, FT3, FT4 increased by 16, 23%, 28, 22% compared to the control group. Moreover, the TSH level in this group decreased (45%)

compared to the LC group; however, its' level was still higher (41%) than the control group (Table 2).

III. Histopathology of the thyroid gland

The follicles of the control group are lined by cubical cells and contained on their center's acidophilic homogenous colloid (Fig 1a).

The follicles in the LC group showed variable activities; some follicles are distended, while others are involuted. The distended follicles are lined by flat cells. Moreover, some follicles are disorganized with wide interfollicular spaces. Furthermore, some follicles have detached follicular cells inside the colloid. The follicular cells are mostly vacuolated. Finally, congested-capillary vessels were detected (Fig 1b-e). With the use of GTE, the thyroid architecture looks almost normal (Fig 1f).

IV. Content of collagen fibres

The fibers' content increased 2-fold in the LC group linked to the control group. Much advance was perceived in the GTE group as its fibers' content was one-fold compared to the control group. The fibers' content in the GTE group was 37% lower liked to the LC group (Fig 2, Table 3).

V. Histochemistry of the thyroid gland

In the control group, the follicular cells displayed strong PAS reactions in colloids and basal laminae (Fig 3a, Table 3). The basal laminae of the follicular cells in the LC group revealed weak PAS reactions. The colloids revealed moderate PAS reactions, while the reactions were absent in the vacuoles of colloids. The disrupted basement membranes displayed discontinuous PAS reaction. The reaction was 64% lower than the LC group (Fig 3b, c, Table 3). After the concomitant treatment with GTE, the PAS reaction was strong in colloids and basement membranes. The reaction was comparable to the control group and was > one-fold higher than the LC group (Fig 3d, Table 3).

VI. Immunohistochemistry of the thyroid gland

The control group displayed positive PCNA follicular cell nuclei (Fig 4a). The LC group displayed an increased number of positive PCNA follicular cell nuclei (6.5-fold higher linked to the control group) (Fig 4b, c, Table 3). With the use of GTE, a two-fold increase in the number of positive PCNA follicular cell nuclei linked to the LC group. The expression in the control and GTE groups was identical (Fig 4d, Table 3).

The control group displayed weak caspase-3 expression of the follicular cell nuclei (Fig 5a). The LC group showed strong expression in the follicular cell nuclei (2-fold higher linked the control group) (Fig 5b, c, Table 3). With the administration of GTE, a one-fold increase in the expression in the cell nuclei linked to the LC group. The expression in the control and GTE groups was similar (Fig 5d, Table 3).

The control group showed weak expression of TNF-α of the follicular cells (Fig 6a). The expression in the LC group was strong (2-fold higher liked to the control group) (Fig 6b, c, Table 4). With the utilization of GTE, a one-fold increase in the expression in the follicular cell linked to the LC group. The expression in the control and GTE groups was similar (Fig 6d, Table 4).

VII. Western blot evaluation of Bax and Bcl2 (Fig 7, Table 3)

The expression of Bax in the LC group increased by 190% (about 2-fold) linked to the control group. The expression in the GTE group was 46% lower linked to the LC group but still higher (57%) liked to the control group.

The expression of Bcl2 in the LC group decreased by 59% linked to the control group. The expression in the GTE group was one-fold higher linked to the LC group, but 15% lower linked to control group.

The Bax/Bcl2 ratio was 8-fold higher linked to the control group. The ratio of the control and GTE groups were comparable.

VIII. Proinflammatory and oxidative markers assessment (Table 4)

IL-6 of the LC group was 5-fold higher linked to the control group. With the use of GTE, IL-6 became 2.5-fold higher linked to the control group.

MDA of the LC group was 171% higher linked to the control group. With the use of GTE, MDA became 49% lower linked to the LC group; however, it was 47% higher liked to the control group.

GSH and SOD of the LC group decreased by 62, 42% linked to the control. With the use of GTE, the antioxidant markers increased by 121, 97% linked to the LC group; however, both markers were 17,16 % higher linked to the control.

DISCUSSION

Under our experimental condition, hypothyroidism induced by lithium was confirmed by decrease the level of thyroid hormones (TT3, TT4, FT3, FT4). LC influences its effects over the thyroid hormones either directly or indirectly via the hypothalamic- pituitary- thyroid axis (Lazarus, 2009).

Moreover, an increase in serum level of TSH occurs mostly secondary to the decreased thyroid hormone secretion. TSH is a thyrotropin hormone that is secreted from the pituitary gland and stimuluses the formation of T3 and T4 (Grossmann et al., 1997). It is the key indicator of thyroid dysfunction (Benhadi et al., 2010). The resultant hypersecretion of TSH specifies the commencement of hypothyroidism as described formerly in patients treated with LC(Kleiner et al., 1999). The diagnostic characteristic of TSH emerges from the inverse linear relations between the serum TSH and FT4 level as tiny changes in T4 levels induces enormous changes in serum TSH (Benhadi et al., 2010, Baloch et al., 2003).

By the end of the work, the BW of the LC rats increased by 21% compared to the control group. The weight gain is typically related to low basal metabolic rates consequence to the hypothyroidism induced by LC (Mackowiak et al., 1999)

The prolonged take of LC is associated with thyroid damage. The colloids are extensively vacuolated and depleted. The follicles either distended or involuted. Moreover, some follicles are disorganized with wide interfollicular spaces and detached follicular cells. Adding, the follicular cells show vacuolations.

The observed thyroid damage in the LC group is mostly multifactorial. Oxidative stress is one of the pathogenic mechanisms through which LC can induce thyroid damage at the cellular level. Many researchers proved the oxidative stress role of LC over many organs such as heart, kidney, and testis (Ossani et al., 2019, Mezni et al., 2017). Oxidative stress is a shift in the balance between oxidants and antioxidants in favor of oxidants (Birben et al., 2012). With the prolonged use of LC, the oxidant marker (MDA) became high (171%), while the antioxidant markers (GSH and SOD) became low (62, 42%).

The resulting oxidative stress of LC creates oxygen free radical (ROS) that reacts with numerous biomolecules in the cell, leading eventually to oxidative damage (McCord, 1993). ROS is scavenged by several cellular defense mechanisms involving non-enzymatic (GSH) and enzymatic (SOD) scavenger mechanisms. SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2 (Husain and Somani, 1998), while GSH offers protection against free radicals, peroxides, and

toxic compounds (Meister, 1994). Prolonged use of LC decreased the activities of the free radical scavenging enzymes SOD and GSH. This consequences in augmented production of the O_2^- and H_2O_2 which in turn outcomes the production of OH^- (Halliwell and Gutteridge, 1988). The generation of OH^- takes part in the observed thyroid toxic damage (Halliwell and Gutteridge, 1988).

Higher lipid peroxidation (higher MDA) , noticed in the LC group, led to distraction of the follicular basement membranes' integrity, and the cytoplasmic enzyme's leakage (Dhouib et al., 2015). Many researchers believed that MDA's level is sufficient proof of oxidative stress (Kurt et al., 2015). So finally, the increased MDA content indicates severe oxidative stress and increased lipid peroxidation.

Sterile inflammation (inflammation in the absence of infection) is the second pathogenic mechanisms through which LC induced thyroid damage at the cellular level. Inflammation was evident by the increase of the pro-inflammatory cytokines (a 2-fold elevation of TNF-α and 5-fold increase of IL-6). TNF-α is accountable for the pathogenesis of increases in ROS and oxidative stress (Kurt et al., 2015). It controls the growth, proliferation, differentiation, and viability of activated leukocytes (Nair et al., 2006). Furthermore, TNF-α elicits the cellular release of cytokines, chemokines, or inflammatory mediators (Nair et al., 2006). So, extreme TNF-α secretion results in thyroid injury indirectly through inducing oxidative stress and directly through inducing apoptosis. The resultant fibrosis was consequent to oxidative stress and inflammation.

Congestion in the vasculature of the thyroid gland was a constant feature in the LC group. It is caused as a result of the increased demand for blood to feed the follicular cells or as a result of the increased thyroid gland's size (El-Mahalaway and El-Azab, 2019). Furthermore, congestion can be part of the previously stated inflammatory process that accompanies LC toxicity. Moreover, prolonged elevation of TSH induces neovascularization and increases the stromal vascularity as TSH acts as a growth factor for the thyroid tissue (Hassanin et al., 2013).

Apoptosis is also a main pathogenic mechanism through which LC induced thyroid damage at the cellular level. Apoptosis is induced by higher levels of MDA and TNF-α (Birben et al., 2012, Kurt et al., 2015). Apoptosis enhances the release of cytokines and ROS which lastly damages thyroid gland (Kurt et al., 2015).

The framework of the apoptotic signal pathway finally converges into a common mechanism driven by caspases(Thornberry and Lazebnik, 1998). Caspase-3 is the principal destroyer of apoptosis, thus sponsoring cell survival (Ma et al., 2014). caspase 3 of the LC group was 2-fold high linked to the control. The caspases mechanism is negatively regulated by Bcl-2 family unit (Adams and Cory, 1998). This family is classified into a family containing Bcl-2, a second family containing Bax, and the third family includes Bik and Bid (Tsujimoto, 1998). The Bcl-2 exerts anti-apoptotic activity by impeding a step that leads to the activation of caspases, while Bax exerts proapoptotic activity(Tsujimoto, 1998). With the use of LC, the expression of Bax increased 2-fold, while the expression of Bcl2 decreased by 59%. The Bax/Bcl2 ratio increased 8-fold. This ratio describes the accountability of the cell to apoptosis (Tsujimoto, 1998).

With prolonged exposure to LC, some follicular cells may pass into irreparable damage and undergo terminal growth arrest or apoptosis. However, other follicular cells may acquire an intrinsic mechanism of death resistance and finally lead to hyperplasia instead of apoptosis. Such hyperplasia was furtherly confirmed morphometrically by a significant increase in the number of PCNA positive follicular cell nuclei (6.5-fold). PCNA is directly correlated with the proliferative state of various tissues (Zhong et al., 2008, Velický et al., 1997).

GTE has a perfect influence on thyroid damage induced by LC. The beneficial impacts of GTE are ascribed to the polyphenolic compounds, particularly the catechins, which constitute 30% of the dry weight of GT leaves (Graham, 1992).

With the use of GTE, the level of thyroid hormones increased, while the TSH level decreased. However, the hormonal levels are still away from the control group. BW in the GTE group was comparable to the control group. Weight improvement is mostly explained by increase basal metabolic rates consequence to regain the normal thyroid function.

Besides, the expression of PCNA in the GTE group was like the control group. This is mostly attributed to the antiproliferative property of GTE (Li et al., 2014).

Apoptosis is muchly improved in the GTE group as the expression of caspase-3 and Bax/Bcl2 ratio were comparable to the control. Moreover, the expression of Bax decreased, while that of Bcl2 increased. Such protective role of GTE could be explained by its antioxidant effect (decrease MDA/increase GSH, SOD). The antioxidant effect of GTE was documented in

many works (Roychoudhury et al., 2017, Peluso and Serafini, 2017). Catechins and theaflavins of GT are responsible for such antioxidant activity(Benzie and Wachtel-Galor, 2011).

Besides, we proved the antifibrotic and anti-inflammatory role of GTE as the collagen fibers content and proinflammatory markers (TNF-α and IL-6) decreased linked to the LC group. The anti-inflammatory and antifibrotic roles of GTE were proved in many literatures (Ohishi et al., 2016, Wang et al., 2019).

There seem to be two major effects of lithium salts in bipolar disorder, i.e. Inhibitory action on inositol monophosphatase and inhibition of glycogen synthase kinase-3 activity. No reports in the works of literature documented any effect of GTE on these enzymes. So, GTE has no harm interfering with lithium action on the brain.

In conclusion, prolonged use of LC results in hypothyroidism, which is accompanied by structural thyroid damage. LC induced its thyroid damage through oxidative stress that prompted sterile inflammation and apoptosis. With the use of GTE, the thyroid gland achieved its structure and function. The protecting role of GTE is through its antioxidant, anti-inflammatory, antiproliferative and antifibrotic effects.

Ethical approval: All relevant international, national, and/or institutional guidelines for the care and use of animals were tracked. The study was permitted by the Ethics Committee, Faculty of Medicine, Cairo University.

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Conflict of interest: none

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Table 1. Body weight in the different groups at the end of study

Group	BW (g) Mean ± SD	Versus group	P value
Control	200±22.5		
LC-treated	245.5±30.8	Control	0.01*
		GET-treated	0.03*
GTE-treated	215.6±18.4	Control	0.1
		LC-treated	0.03*

The BW at the beginning of the study was 160.5±17.1 g.

*= P-value significant

Group		TSH	TT3 (ng/ml)	FT3 (pmol/L)	TT4 (µg/dl)	FT4 (pmol/L)
Control	Mean ±SD	**3.6±0.3**	**6.2±0.5**	**16.7±0.8**	**21.2±1.0**	**32.9±3.2**
LC-treated	Mean ±SD	**9.4±1.3**	**4.4±0.2**	**7.7±1.2**	**14.4±1.7**	**16.3±3.0**
	Versus control	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
	Versus GTE-treated	< 0.001*	0.01*	< 0.001*	0.055	0.002*
GTE-treated	Mean ±SD	**5.1±0.3**	**5.2±0.4**	**12.0±0.8**	**17.2±2.1**	**25.4±3.0**
	Versus control	< 0.001*	0.002*	< 0.001*	0.007*	0.007*
	Versus LC-treated	< 0.001*	0.01*	< 0.001*	0.055	0.002*

Table 2. Thyroid function tests at the end of the study

*= p-value significant

Group		Content of collagen fibres	Optical density of PAS reaction	Caspase 3 positive cells (% of total cells)	PCNA positive cells (% of total cells)	Bax	Bcl2	Bax/Bcl2 ratio
Control	Mean ±SD	**18.2±5.9**	**0.31±0.06**	**4.0±1.6**	**12.8±1.9**	**0.83±0.05**	**2.73±0.16**	**0.27±0.05**
LC-treated	Mean ±SD	**55.2±8.2**	**0.11±10.06**	**30.0±7.9**	**38.8±6.3**	**2.44±0.16**	**1.11±0.05**	**2.42±0.39**
	Versus control group	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
	Versus GTE-treated group	0.003*	0.005*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
GTE-treated	Mean ±SD	**37.4±5.0**	**0.24±0.04**	**12.6±2.8**	**23.2±3.3**	**1.31±0.07**	**2.32±0.706**	**0.54±0.03**
	Versus control group	0.002*	0.144	0.052	0.007*	< 0.001*	< 0.001*	0.259
	Versus LC-treated group	0.003*	0.005*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Table 3. Collagen fibres, PAS reaction, apoptotic and proliferative markers

*= p-value significant

Group		MDA (nmol/mg tissue protein)	GSH (μg/mg tissue protein)	SOD (nmol/mg tissue protein)	IL-6 (pg/mg tissue protein)	TNF-alpha (pg/mg tissue protein)
Control	Mean ±SD	**1.53±0.16**	**1.89±0.05**	**7.1±0.46**	**82.0±7.81**	**64.6±10.04**
LC-treated	Mean ±SD	**4.16±0.41**	**0.7±0.14**	**2.98±0.4**	**374.0±24.65**	**388.0±16.84**
	Versus control	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
	Versus GTE-treated	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
GTE-treated	Mean ±SD	**2.25±0.09**	**1.55±0.12**	**5.9±0.3**	**158.8±14.7**	**243.00±30.02**
	Versus control	0.003*	0.01*	0.01*	< 0.001*	< 0.001*
	Versus LC-treated	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Table 4. Inflammatory and oxidative/antioxidative markers

*= p-value significant

Figure 1. (**a):** Normal follicles of the control rats. Note simple cuboidal follicular cells (arrows) surrounding homogenous acidophilic colloid (co). **b-e:** Thyroid follicles of LC group. **(b):** Some follicles are distended (D), others are involuted (I). Note damaged and disorganized follicles (encircle) with a large interfollicular space (S). Note follicular cells with vacuolated cytoplasm (arrowheads). **(c):** Markedly distended thyroid follicle (D). Note congested-capillary vessel (CV) and follicular cells with vacuolated cytoplasm (arrowheads). **(d):** Loss of normal thyroid architecture. Most of the follicles have shredded epithelial lining (curved arrows) and have no colloid. Note disorganized follicles (encircle) with a large interfollicular space (S). **(e):** Thyroid follicles (F) with vacuolated cells (arrowheads). Note congested-capillary vessel (CV), interfollicular spaces with extravasated blood (c). Notice part of the parathyroid gland (Pa). **(f):** Nearly normal thyroid architecture of the GTE rats. Note follicular cells with vacuolated cytoplasm (arrowheads). H & E X 400.

Figure 2. (**a):** The collagen fibers (arrows) between thyroid follicles in the control. **(b, c):** Increased collagen fibers (arrows) between thyroid follicles in the LC rats. (**c):** Negligible collagen fibers (arrows) between thyroid follicles in the GTE rats. Masson's trichrome, X 400.

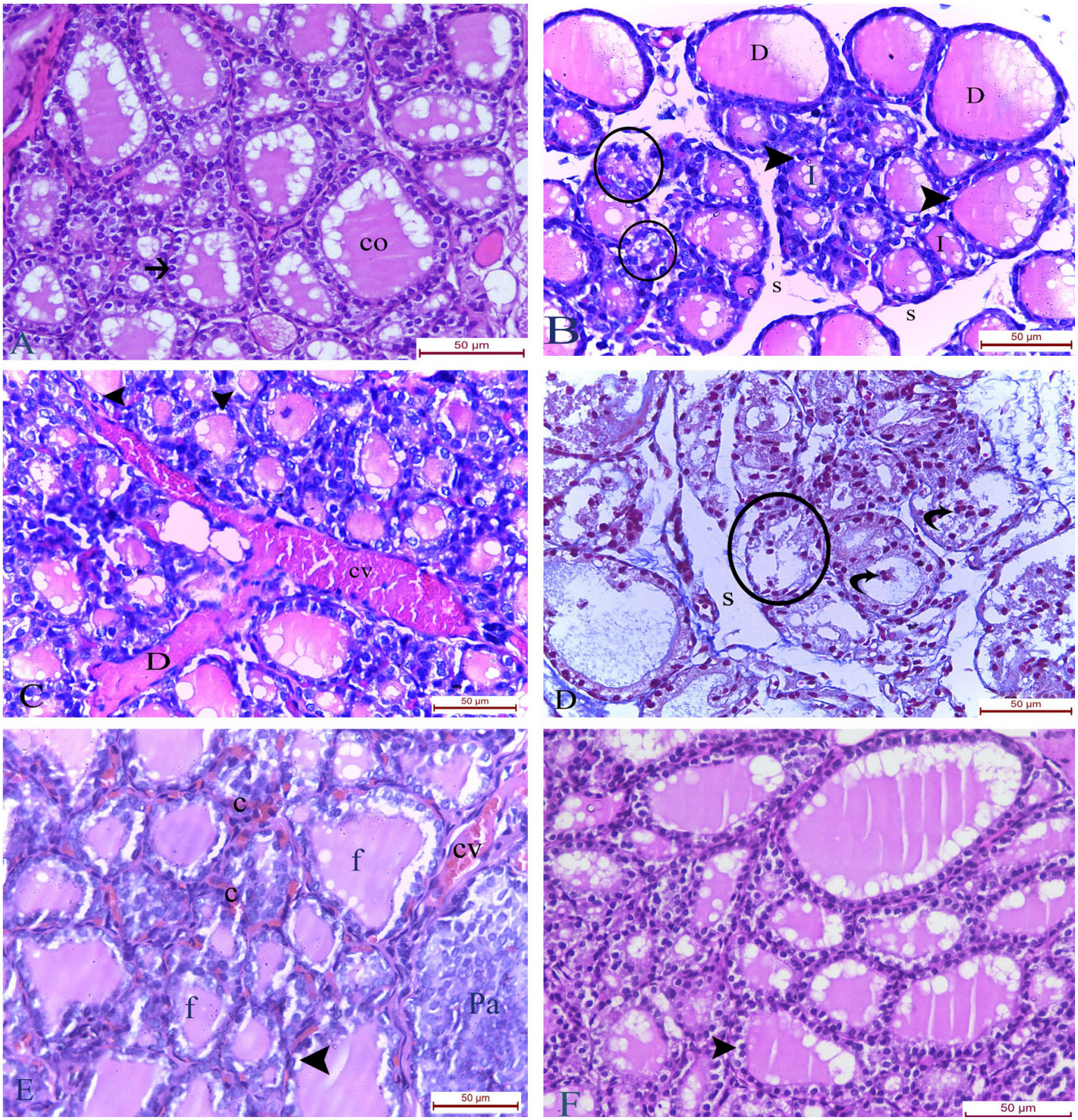
Figure 3. (a): Strong PAS-positive reaction in the colloid (co) and basement membranes (arrows) of the follicles with a negative reaction in the peripheral vacuoles of colloids (v) in the control group. **(b, c):** Weak PAS reaction in the basement membranes (arrows) of the follicles in the LC group. The PAS reaction was moderate in colloids (co), while it was absent in peripheral vacuoles of colloids (v). Note discontinuous PAS-positive reaction in the disrupted basement membranes (arrowheads). **(d):** Strong PAS-positive reaction in colloids (co) and basement membranes (arrows) of the follicles with a negative reaction in the minute peripheral vacuoles of colloids (v) in the GTE group. PAS X 400.

Figure 4. ***(*****a):** The number of PCNA positive cells (arrows) in the control rats. **(b, c):** Apparent increase in the number of PCNA positive cells (arrows) in the LC rats. (**d):** Slight increase in the number of PCNA positive cells (arrows) in the GTE rats. PCNA X 400.

Figure 5. ***(*****a):** Control group exhibited weak caspase-3 expression in the follicular cell nuclei (arrows). **(b, c):** The LC group showed strong expression in the follicular cell nuclei (arrows). (**d):** Slight expression in the follicular cell nuclei (arrows) in the GTE group. Caspase-3 X 400.

Figure 6. ***(*****a):** Control group displayed weak expression of TNF-α in the follicular cell nuclei (arrows). **(b, c):** The LC group showed increased expression of TNF-α in the follicular cell nuclei (arrows). (**d):** Slight increase expression of TNF-α in the follicular cell nuclei (arrows) in the GTE group. TNF-α X 400.

Figure 7. Western blot assay of Bax and Bcl2. Bax was higher in the LC group linked to the control. Bcl2 was lower in the GTE group linked to the control. Beta-actin was used as a control.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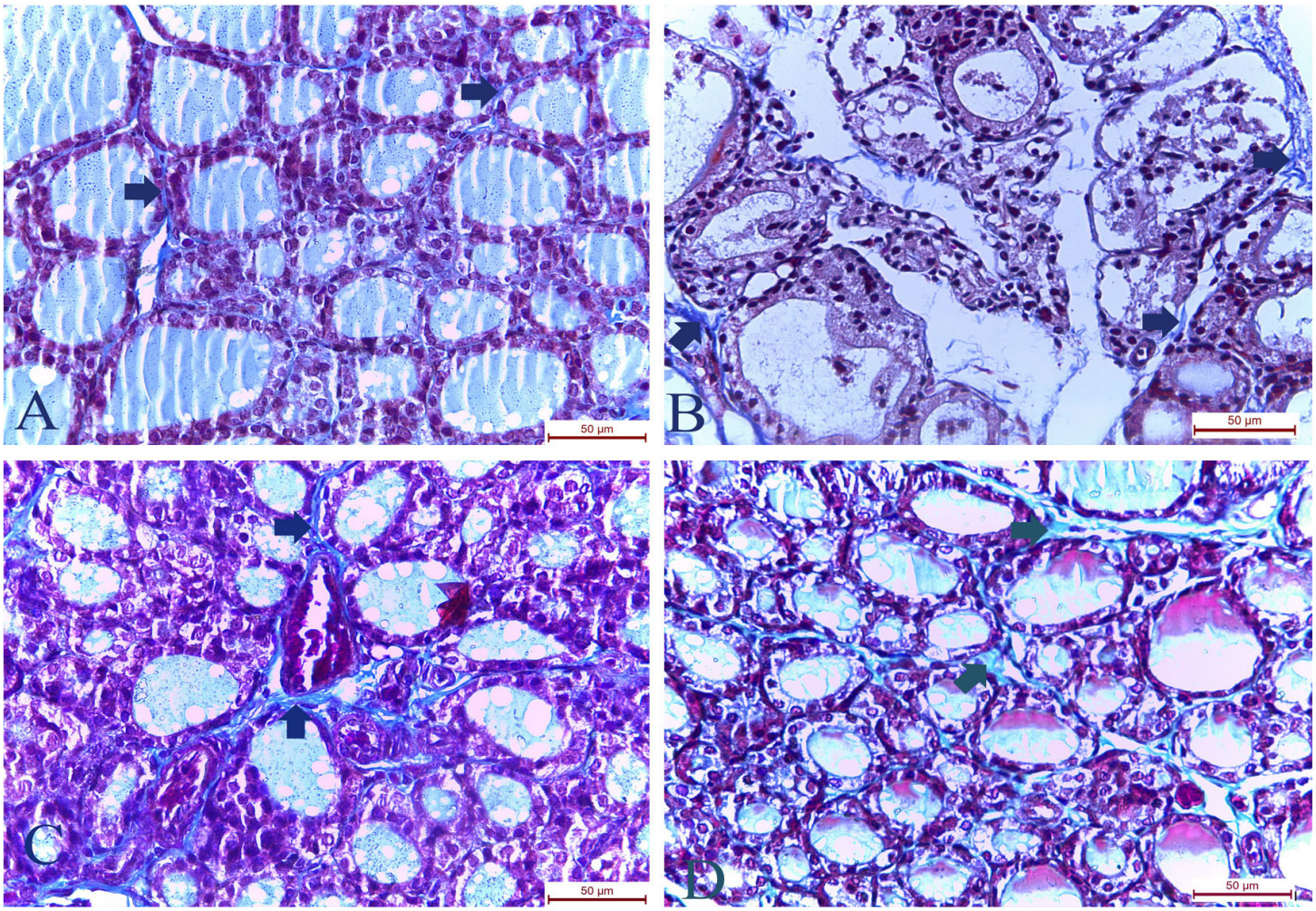
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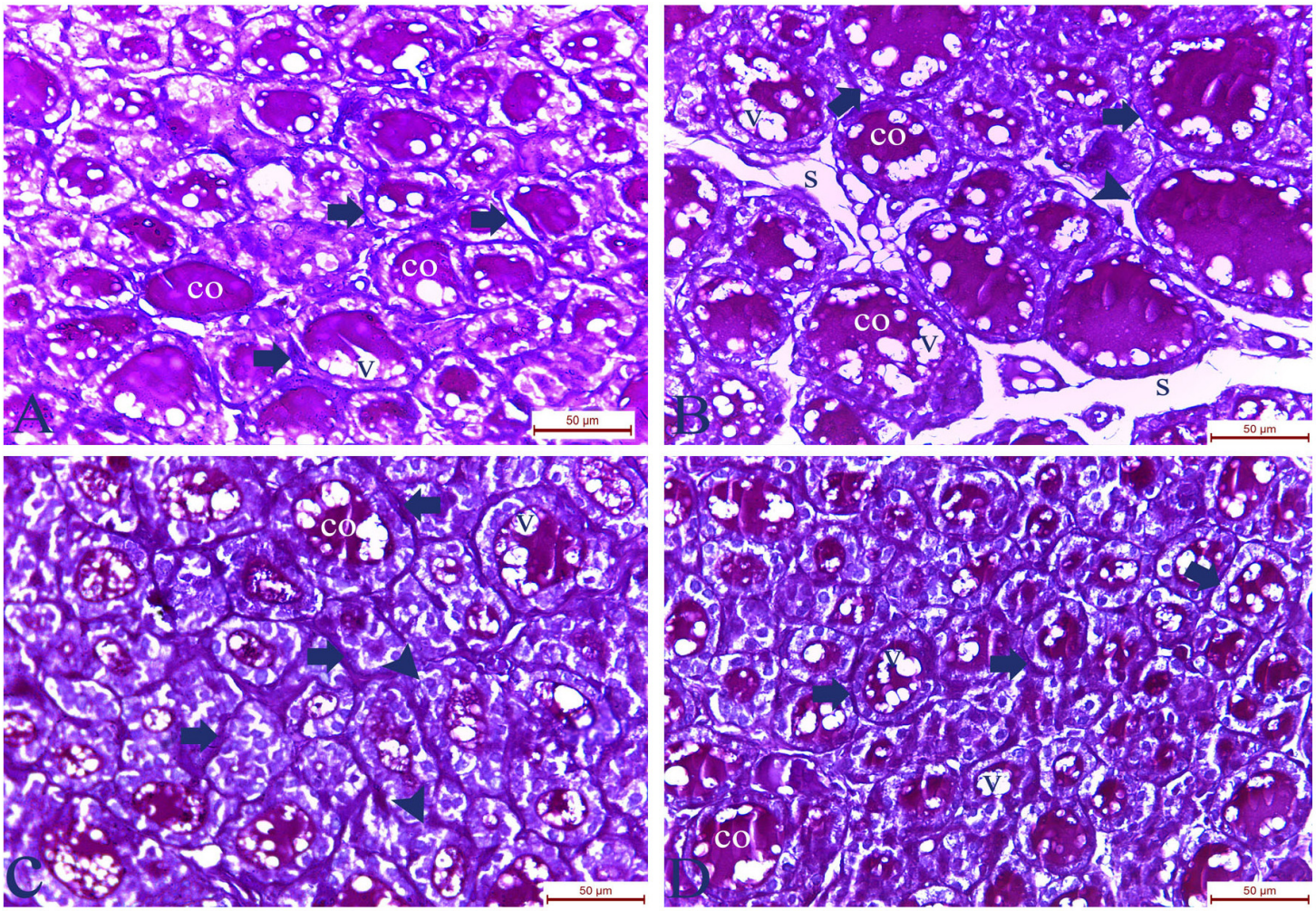
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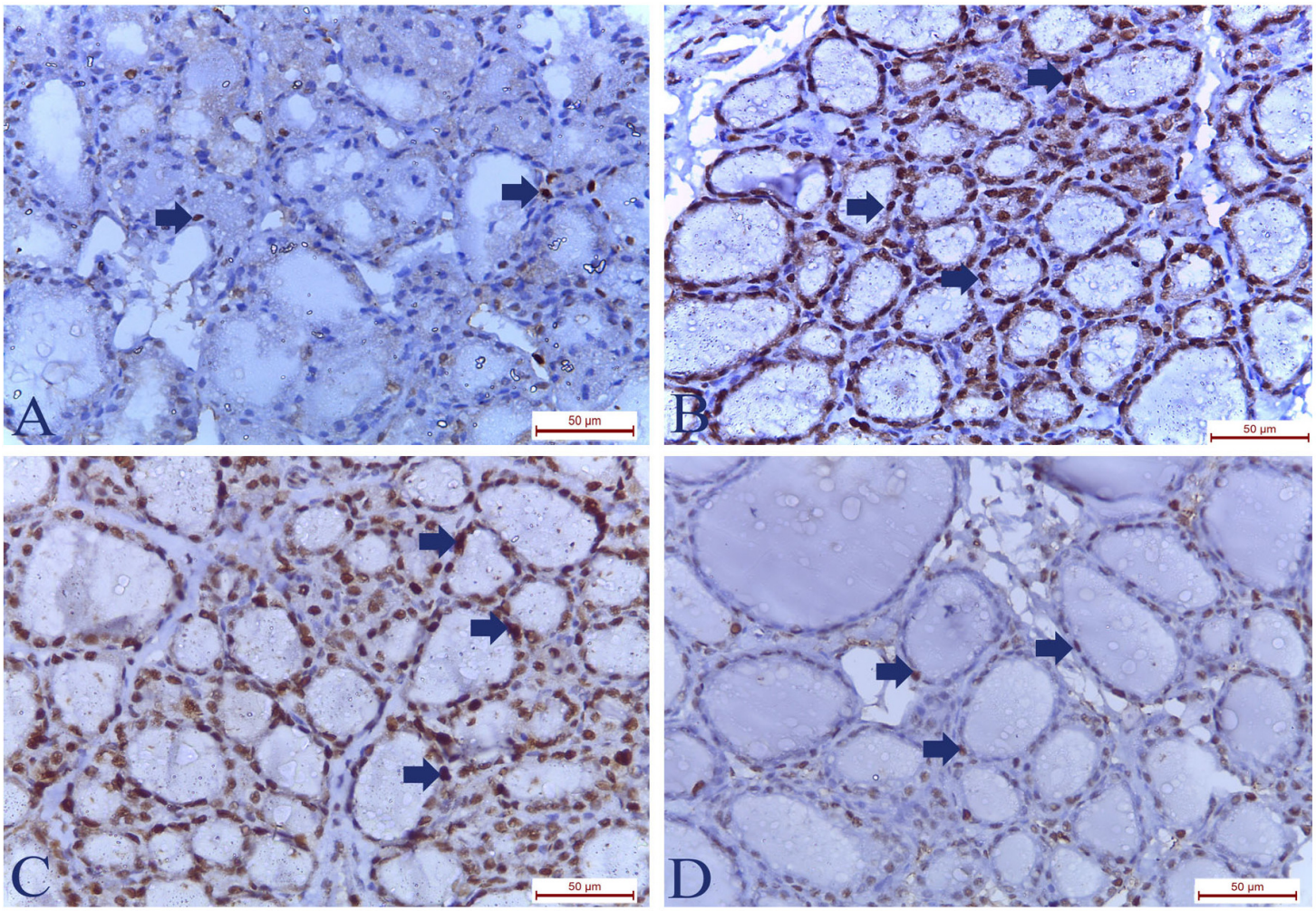
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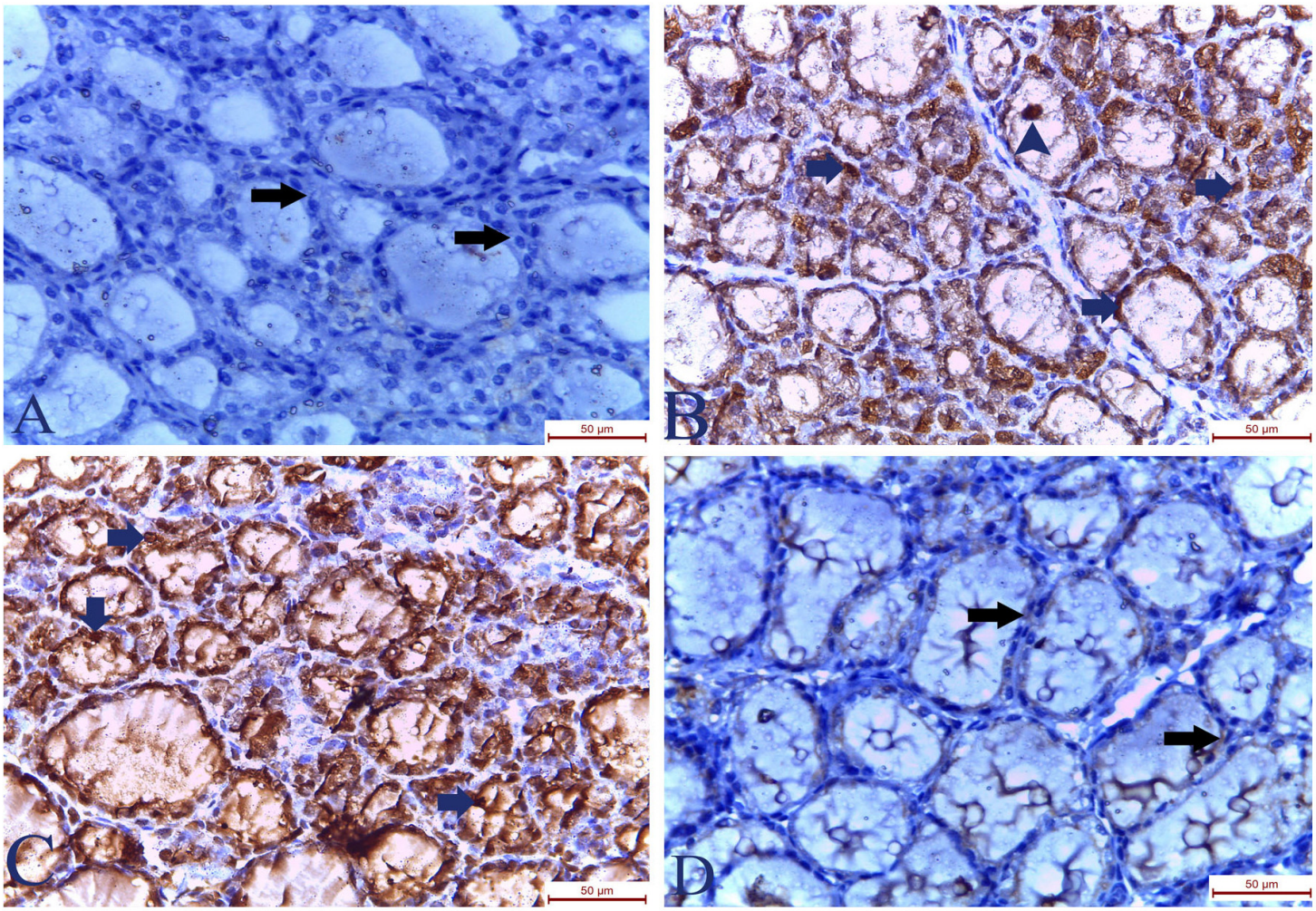
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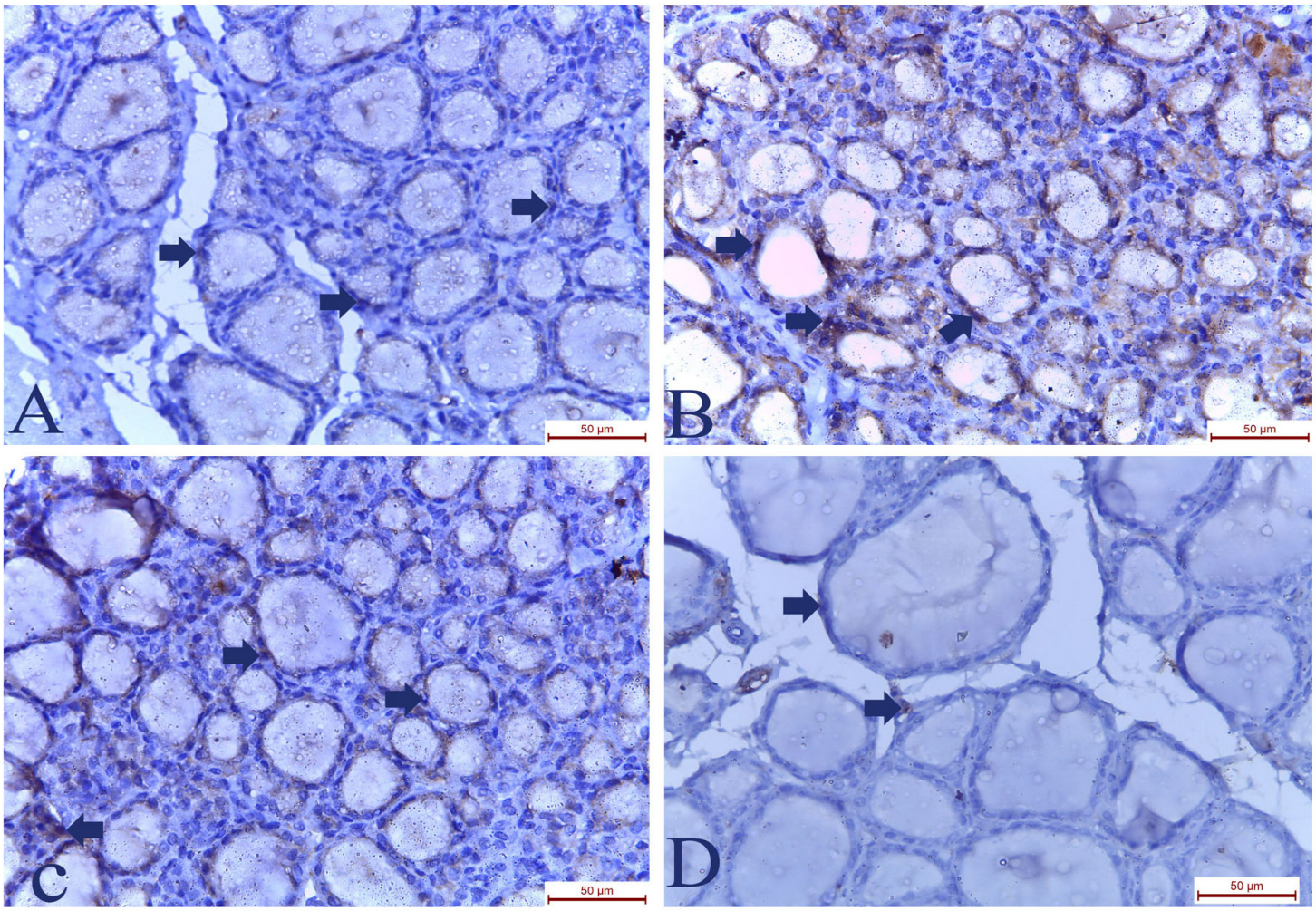
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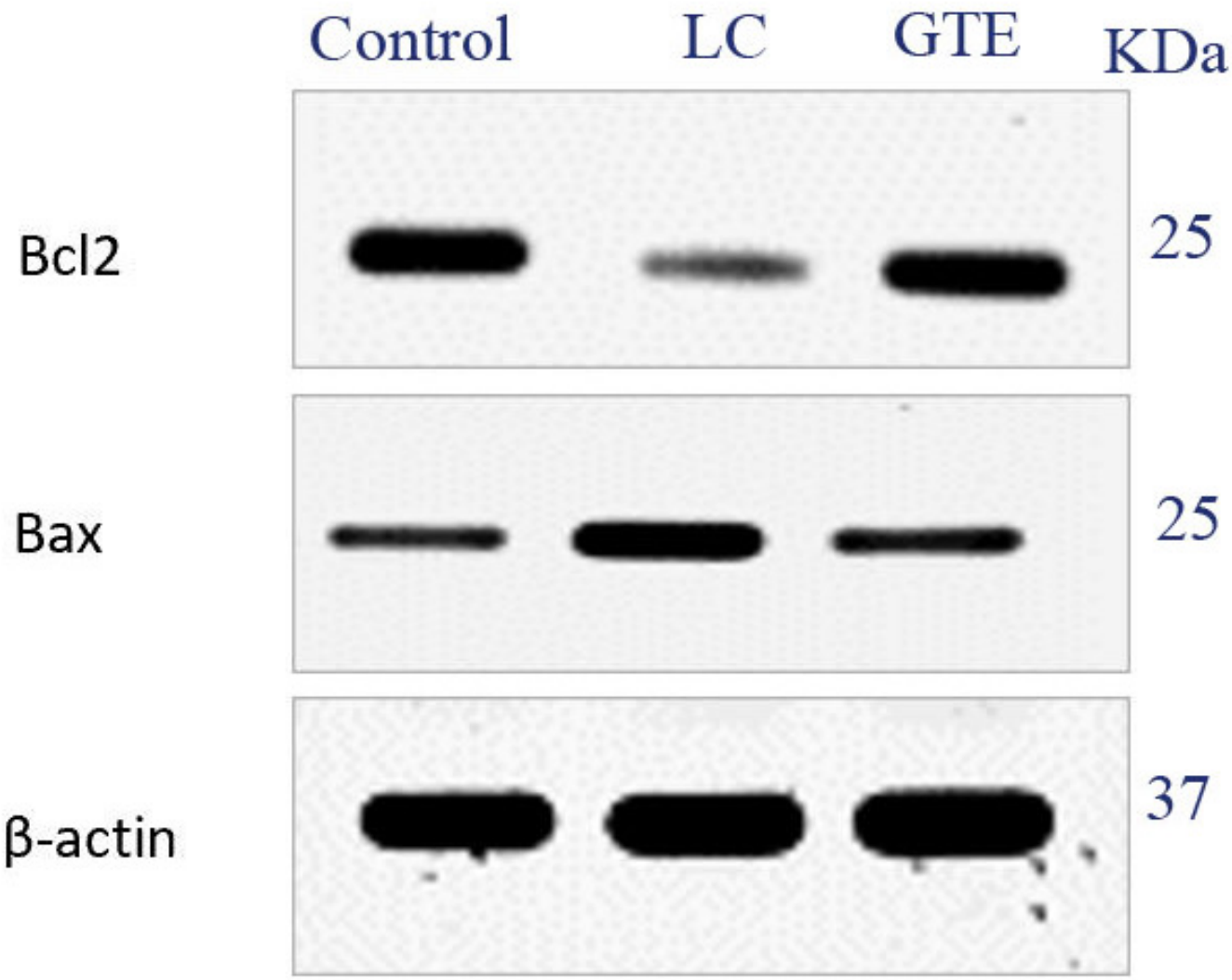
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